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Degradation and Turnover of Peroxisomes in the Yeast *Hansenula polymorpha* Induced by Selective Inactivation of Peroxisomal Enzymes

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Abstract. Inactivation of peroxisomal enzymes in the yeast *Hansenula polymorpha* was studied following transfer of cells into cultivation media in which their activity was no longer required for growth. After transfer of methanol-grown cells into media containing glucose – a substrate that fully represses alcohol oxidase synthesis – the rapid inactivation of alcohol oxidase and catalase was paralleled by a disappearance of alcohol oxidase and catalase protein. The rate and extent of this inactivation was dependent upon conditions of cultivation of cells prior to their transfer. This carbon catabolite inactivation of alcohol oxidase was paralleled by degradation of peroxisomes which occurred by means of an autophagic process that was initiated by the formation of a number of electron-dense membranes around the organelles to be degraded. Sequestration was confined to peroxisomes; other cell-components such as ribosomes were absent in the sequestered cell compartment. Also, cytochemically, hydrolytic enzymes could not be demonstrated in these autophagosomes. The vacuole played a major role in the subsequent peroxisomal breakdown since it provided the enzymes required for proteolysis. Two basically similar mechanisms were observed with respect to the administration of vacuolar enzymes into the sequestered cell compartment. The first mechanism involved incorporation of a small vacuolar vesicle into the sequestered cell compartment. The delimiting membrane of this vacuolar vesicle subsequently disrupted, thereby exposing the contents of the sequestered cell compartment to vacuolar hydrolases which then degraded the peroxisomal proteins. The second mechanism, observed in cells which already contained one or more autophagic vacuoles, included fusion of the delimiting membranes of an autophagosome with the membrane surrounding an autophagic vacuole which led to migration of the peroxisome inside the latter organelle. Peroxisomes of methanol-grown *H. polymorpha* were degraded individually. In one cell 2 or 3 peroxisomes might be subject to degradation at the same time, but they were never observed together in one autophagosome. However, fusions of autophagic vacuoles in one cell were frequently observed. After inhibition of the cell's energy-metabolism by cyanide ions or during anaerobic incubations the formation of autophagosomes was prevented and degradation was not observed.

Key words: Peroxisome – Degradation – Autophagy – Catabolite inactivation – Alcohol oxidase – Catalase – Cytochemical staining – Ultracryotomy – *Hansenula polymorpha*

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In the methylotrophic yeast *Hansenula polyorpha*, peroxisomes generally develop in response to certain changes in the cell's environment. They originate from pre-existing organelles and develop by means of growth and division (Veenhuis et al. 1978a, 1979). The ultimate shape of the organelles, their number, substructure and physiological function depends entirely upon environmental conditions. They may be involved in carbon metabolism (i.e. during growth on methanol) and/or nitrogen metabolism (i.e. during growth in the presence of organic nitrogen sources such as methylamine, urate and D-amino acids) (van Dijken 1976; Veenhuis et al. 1983; Zwart et al. 1980). When cells are grown on these carbon- and/or nitrogen sources, the peroxisomes invariably contain key enzymes involved in the oxidative metabolism of these compounds. During exponential growth of *H. polymorpha* in batch cultures on methanol, large crystalloids develop in the peroxisomes, due to crystallization of alcohol oxidase protein (Veenhuis et al. 1981b). In cells of methanol-limited chemostat cultures completely crystalline peroxisomes generally of cuboid shape were present. Under the latter conditions individual cells may contain up to 20 peroxisomes, which together make up approximately 80% of the cytoplasmic volume (Veenhuis et al. 1978a). In contrast, during exponential growth of *H. polymorpha* in media containing glucose and ammonium sulphate, a proliferation of peroxisomes is not observed. Under these conditions, when the synthesis of alcohol oxidase is fully repressed (Eggeling and Sahm 1978; Egli 1980; Egli et al. 1980), the cells generally contain a single small peroxisome located in close proximity to the cell wall (Veenhuis et al. 1979).

Exposure of methanol-grown cells to glucose- or ethanol-excess conditions leads to a rapid inactivation of both alcohol oxidase and catalase (Bormann and Sahm 1978; Bormann 1980; Veenhuis et al. 1978b), associated with a loss of alcohol oxidase protein (Bruinenberg et al. 1982). This is consistent with electron microscopical observations which revealed that the disappearance of activity of this peroxisomal enzyme was paralleled by a decrease in number and volume fraction of the peroxisomes in the cells. These observations (Veenhuis et al. 1978b) also suggested that the vacuole, known as the main compartment of hydrolytic activity in yeasts (Wiemken et al. 1979; Wolf and Holzer 1978), might be involved in the degradation of peroxisomes. However, the mechanism of

peroxisomal degradation, in particular the way in which the vacuole is involved in this process, remained unclear.

In the present communication results are presented of a biochemical and ultrastructural study of the inactivation of alcohol oxidase and its correlation to peroxisomal degradation in the yeast *H. polymorpha* after transfer of cells into media in which the activity of the enzyme was no longer required for growth. It is shown that the extent and rate of enzyme inactivation is dependent upon the cultivation conditions prior to and after the transfer. Degradation of peroxisomes was only observed under conditions in which the synthesis of alcohol oxidase was partly or completely repressed and occurred by means of an autophagic process. The subcellular mechanism of this process is described.

Materials and Methods

Organism and Cultivation. All experiments were performed with the yeast *Hansenula polymorpha* de Morais et Maya CBS 4732. The organism was grown in a methanol-limited chemostat at 37° C in the mineral medium of van Dijken et al. (1976) containing 0.5 % (v/v) methanol as the carbon source.

Transfer Experiments. Chemostat-grown cells of *H. polymorpha*, grown at different dilution rates ($D = 0.02$; $D = 0.07$; $D = 0.09$; $D = 0.15\,h^{-1}$) were placed in 1 l Erlenmeyer flasks, containing 300 ml of the mineral medium described previously (Veenhuis et al. 1978a), supplemented with 0.25 % (w/v) glucose, 0.5 % (v/v) glycerol, 0.5 % (w/v) dihydroxyacetone or 0.5 % w/v deoxyglucose. The cultures were inoculated to an optical density (OD_{663}) of approximately 0.5 and incubated at 37° C. Inhibition of energy metabolism was obtained by the addition of potassium cyanide (KCN); (6 mM final concentration). Incubations in the absence of glucose served as controls.

Enzyme Assays. Cell-free extracts were prepared by sonification of whole cells (van Dijken et al. 1976). Alcohol oxidase activity was determined with a Clarktype Oxygen Electrode (Biological Oxygen Monitor, Yellow Springs Instrument Co., USA) by the methods described by van Dijken et al. (1976). Catalase was assayed by the spectrophotometric method of Lück (1963). The activity of alcohol oxidase is expressed as $\mu mol\ O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$, catalase activity is expressed as $\Delta E_{240} \cdot min^{-1} \cdot mg\ protein^{-1}$. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

High Performance Liquid Chromatography. A quantitative determination of alcohol oxidase and catalase was performed in cell-free extracts of *H. polymorpha* as described elsewhere (Bruinenberg et al. 1982). Alcohol oxidase protein was measured at 280 nm; FAD and the heme component of catalase at 436 nm.

Preparation of Spheroplasts. Spheroplasts were prepared by treatment of whole cells with Zymolyase (Kitamura et al. 1971) according to the procedure of Osumi et al. (1975).

Cytochemical Staining Techniques. The cytochemical staining experiments were performed on glutaraldehyde fixed cells or spheroplasts. Catalase activity was demonstrated with diaminobenzidine (DAB) and H_2O_2 or with DAB and methanol as the endogenous source of H_2O_2 (Veenhuis et al. 1976). Alcohol oxidase, acid phosphatase and glucose-6-phosphatase activity were demonstrated using $CeCl_3$ as the H_2O_2- or orthophosphate-capturing agent, respectively, according to methods described previously (Veenhuis et al. 1976, 1980a).

Preparation of Ultrathin Cryosections. For ultracryotomy the cells were fixed in 6 % glutaraldehyde in 0.1 M Na-cacodylate buffer pH 7.2 for 15 min at 0° C. Sections were cut with a glass knife using an LKB-cryokit according to the techniques described by Tokuyashu (1978). The sections were collected on Formvar-carbon coated grids, washed with 0.1 M ammonium acetate and water and subsequently stained with 0.5 % uranyl acetate. Octadecanol, dissolved in 100 % hexane, was used as a wetting agent (Gordon 1972).

Fixation and Postfixation Techniques. Whole cells were fixed with 1.5 % $KMnO_4$ for 20 min at room temperature. Spheroplasts were fixed in 6 % glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 30 min at 0° C. Postfixation of spheroplasts – also after cytochemical staining techniques – was performed in a solution of 1 % OsO_4 and 2.5 % $K_2Cr_2O_7$ in 0.1 M cacodylate buffer pH 7.2 for 45 min at room temperature. After dehydration in a graded alcohol series, the material was embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300 without further staining.

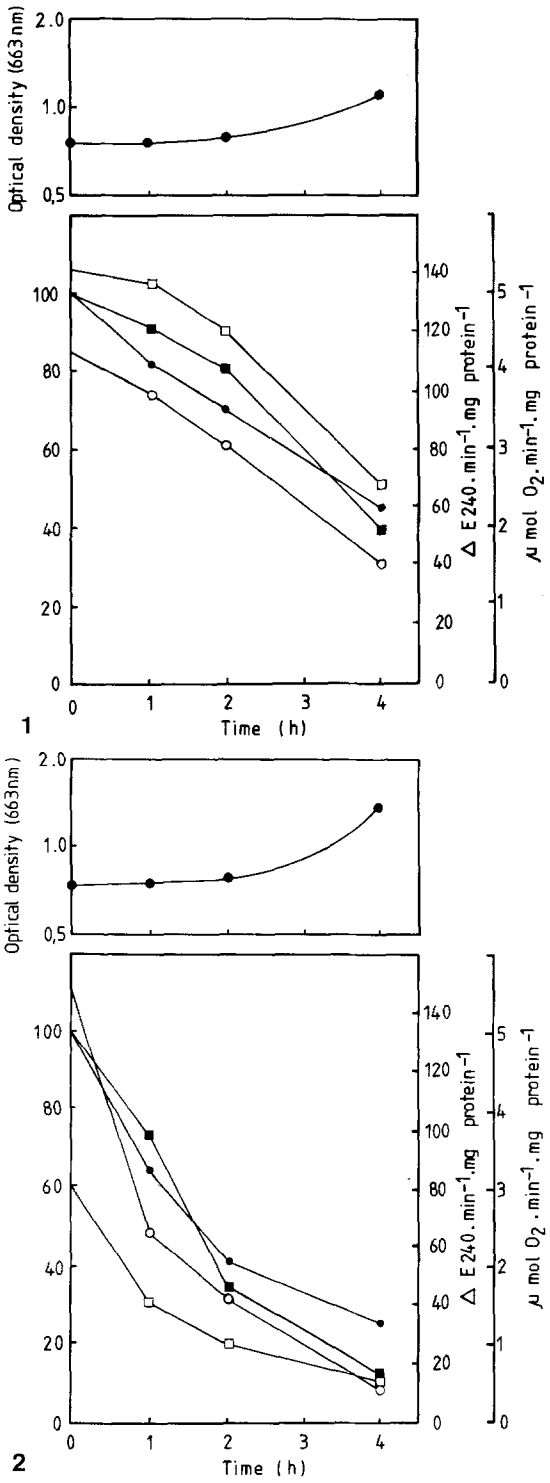
Results

Biochemical Experiments

Transfer of cells of *Hansenula polymorpha*, grown in methanol-limited chemostat cultures into batch cultures containing excess glucose, resulted in a rapid decrease of both alcohol oxidase and catalase activity (Figs. 1, 2) which could not be accounted for by dilution of existing enzyme activity as a result of growth. The kinetics of the observed enzyme inactivation were dependent upon the cultivation conditions extant during growth of the cells on methanol prior to the transfer in that the initial rate of inactivation increased with increasing growth rates (Figs. 1, 2). The rate of adaptation of cells to growth on glucose was similarly influenced by their previous growth rate.

The relative amounts of enzyme protein were determined by high-performance liquid chromatography (HPLC) (Bruinenberg et al. 1982). The results, as calculated from peak heights at 280 nm, showed that the observed decrease in alcohol oxidase activity after transfer of cells to glucose was paraleled by a disappearance of alcohol oxidase protein (Figs. 1, 2). Similar conclusions were drawn on the basis of the flavine prosthetic group of alcohol oxidase (Results not shown, see also (Brunenberg et al. 1982) and the heme component of catalase (Figs, 1, 2).

The rate of enzyme inactivation was also dependent on the nature of the carbon source present in the new environment. Transfer of cells to glycerol – a substrate which partly represses alcohol oxidase synthesis – reduced the rate of inactivation of alcohol oxidase and catalase to approximately 50 % of the values observed after an identical transfer to glucose. Similar results were obtained when dihydroxyacetone was used as the carbon source in such experiments. After transfer of cells to deoxyglucose, a substrate which cannot be metabolized by *H. polymorpha*, the rapid inactivation of alcohol oxidase and catalase was not observed



Figs. 1 and 2. Growth, enzyme inactivation and decrease in enzyme protein after transfer of cells of *Hansenula polymorpha*, grown in a methanol-limited chemostat at $D = 0.02$ (Fig. 1) or $D = 0.12$ (Fig. 2), into glucose-containing media. The decrease in enzyme protein is expressed as percentage of the original values at $t = 0$. (●——●) alcohol oxidase activity; (○——○) catalase activity; (■——■) alcohol oxidase protein (determined by HPLC at 280 nm); (□——□) catalase protein (determined by HPLC as heme at 436 nm)

(Table 1). Inactivation of these enzymes was also prevented during anaerobic incubation of cells on glucose (Table 1). Under aerobic conditions in the presence of KCN, a rapid inactivation of alcohol oxidase, but not of catalase, was

Table 1. Inactivation and decrease in specific activity and amount of protein of alcohol oxidase and catalase in methanol-limited cells of *Hansenula polymorpha* 4 h after transfer of cells to various environmental conditions. Data are expressed as a percentage of the values originally present. Enzymic protein was determined by HPLC and calculated from peak heights at 280 nm (alcohol oxidase) and 436 nm (catalase-bound heme)

Addition to mineral media	Alcohol oxidase		Catalase	
	specific activity	protein	specific activity	protein
(Control without additions)	90	95	91	96
Glucose (aerobic)	20	11	20	37
Deoxyglucose	98	–	96	–
Glucose (anaerobic)	102	104	92	99
Glucose + KCN (6 mM)	23	98	101	130
KCN (6 mM)	13	102	103	107

– not determined

observed (Table 1). However, similar patterns of enzyme inactivation were obtained when glucose was omitted from such media. HPLC analysis of cell-free extracts showed that the decrease of alcohol oxidase activity in cyanide-containing media was not associated with a decrease in amount of alcohol oxidase protein but with a decrease in the amount of the chromophoric group of alcohol oxidase, FAD (Table 1).

Electron Microscopical Observations

Previously (Veenhuis et al. 1978b) we have shown that the rapid inactivation of alcohol oxidase and catalase, which occurred after the addition of excess glucose to exponentially growing batch cultures of *Hansenula polymorpha* on methanol, is paralleled by the degradation of peroxisomes present in the cells. The subcellular mechanism of the degradation process has now been studied in detail using different electron-microscopical techniques in experiments in which methanol-limited cells, known to contain high numbers of peroxisomes (Fig. 3), were transferred to glucose-containing batch cultures. The first visible ultrastructural change observed in the cells following their transfer, as deduced from thin sections of $KMnO_4$-fixed cells, was the sequestration of individual peroxisomes by a number of electron-dense membranes of approximately 60 – 70 Å in thickness, which closely surrounded these organelles (Figs. 3, 4A, B). These membranes most probably are newly formed since no evidence was obtained for enwrapping of peroxisomes by existing membranes (compare Fig. 3). The number of membrane layers surrounding one peroxisome varied considerably and ranged from 2 to up to 12. During their development the membranes were often observed in close association with various cytomembranes such as those of mitochondria (Fig. 3), ER, nuclear envelope (Fig. 5), tonoplast and – infrequently – with specialized Golgi-like membranous structures, filled with electron-dense material (Fig. 6). Freeze-etch preparations of such cells showed that the electron-dense membranes had characteristic smooth fracture faces, comparable to those of the peroxisomal membranes (Veenhuis et al. 1978b; compare also Fig. 18). Cytochemically, the activity of hydrolytic enzymes such as acid phosphatase and glucose-6-phosphatase could not be demonstrated in the sequestered cell compart-

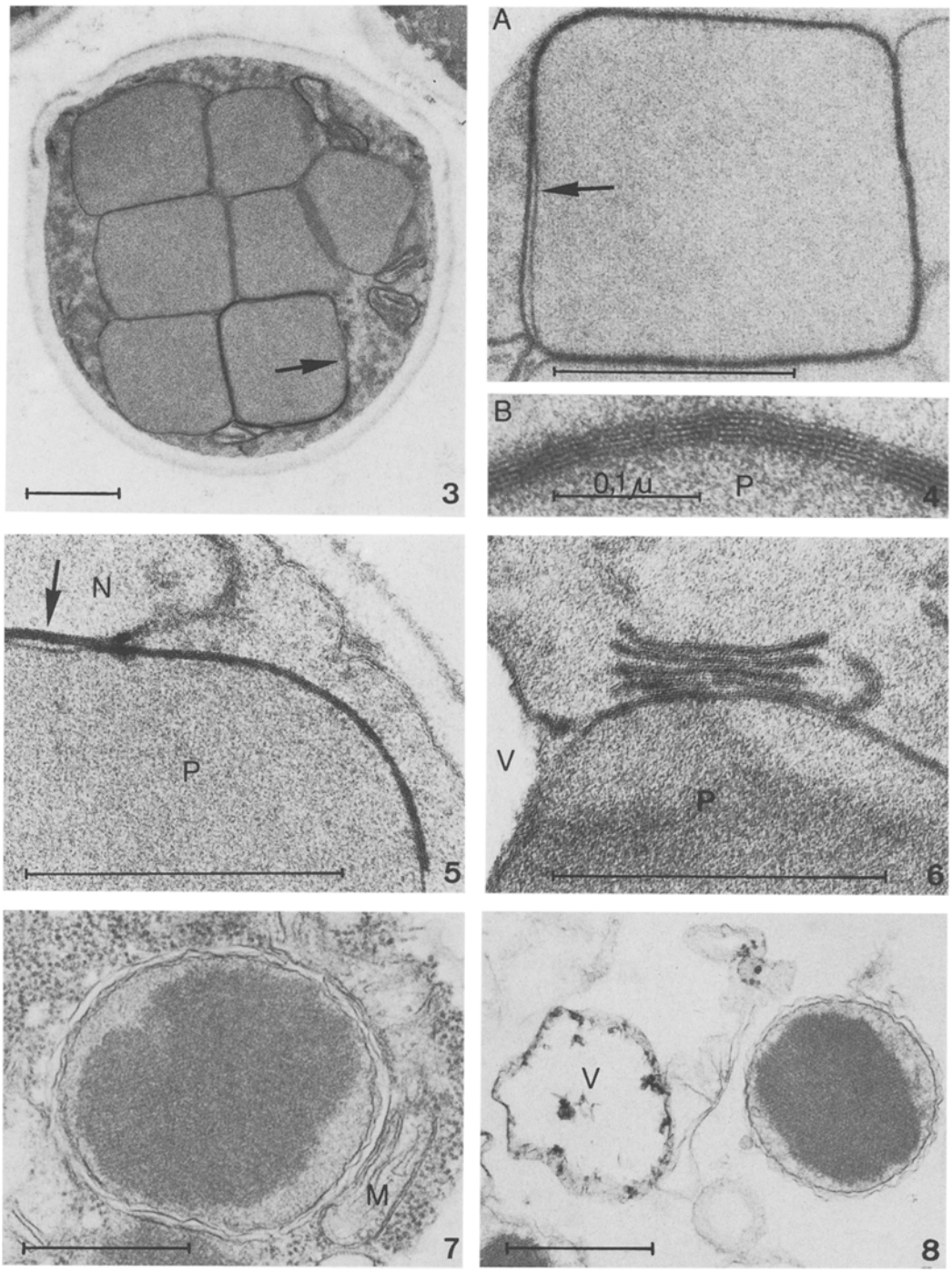


Fig. 3. Survey of a cell from a methanol-limited chemostat culture shortly after transfer to glucose medium. From the cluster of peroxisomes the bottom right organelle is partly sequestered from the cytosol by electron-dense membranes (arrow indicates the unclosed part). Note the close association with a mitochondrion ($D = 0.07$; $t = 20$; $KMnO_4$)

Figs. 4–8. Details of cells, 30 min after their transfer into glucose media. Figure 4 shows electron-dense membranes, surrounding one individual peroxisome from a larger cluster, as visualized after fixation with $KMnO_4$. (Arrow indicates the original peroxisomal membrane). The inset shows a high magnification of a number of membranous layers ($D = 0.02$). Figures 5 and 6 show details of peroxisomes, partially sequestered by electron-dense membranes. The developing membranes are seen in association with the nuclear envelope (Fig. 5; arrow) or a specialized Golgi-like membranous structure, filled with electron-dense material (Fig. 6). Figure 7 shows the appearance of an autophagosome after glutaraldehyde/OsO_4-fixation. The apparent swelling of the organelle is probably due to the fixation procedure. Note the difference in contrast compared to fixation with $KMnO_4$. Figure 7 also demonstrates the absence of other cell components including ribosomes in the autophagosomes. Ribosomes are also absent on the surrounding membranes. The latter is also evident in Fig. 8, showing a section through an isolated autophagosome and a vacuole after cytochemical demonstration of acid phosphatase activity with the Ce^{3+}-technique. Reaction products are localized in the vacuole, but absent in the autophagosome (Fig. 7, 8: $D = 0.12$)

All electron micrographs are taken from cells of *Hansenula polymorpha*, grown in methanol-limited chemostats and subsequently transferred into glucose-containing batch cultures. Dilution rates (D) are given in h^{-1}, the incubation time (t) in the glucose media in min. Abbreviations: *AV* autophagic vacuole; *M* mitochondrion; *N* nucleus; *P* peroxisome; *V* vacuole. The bar represents 0.5 µm

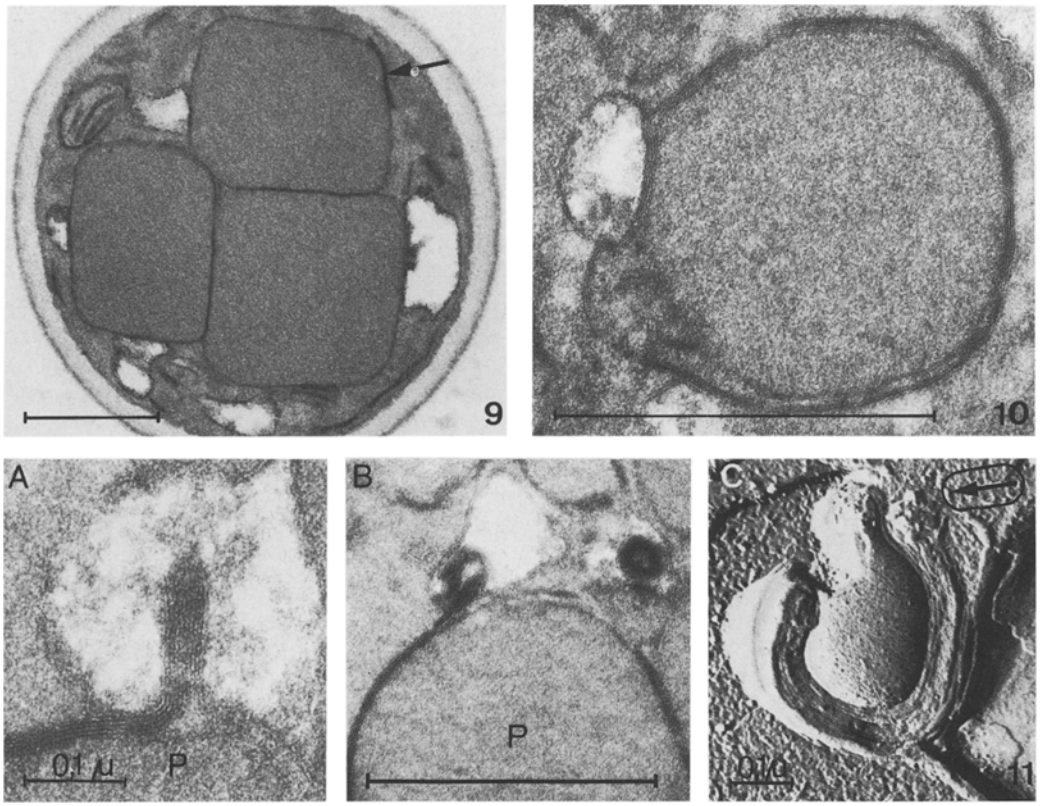


Fig. 9. Survey of a cell, showing the fragmented vacuole, characteristic for methanol-limited cells after transfer to glucose. The arrow indicates the initiation of membrane synthesis leading to sequestration of one of the peroxisomes ($D = 0.12$; $t = 20$; $KMnO_4$)

Fig. 10. Detail of a cell illustrating the association of a small vacuolar vesicle with the electron-dense membranes ($D = 0.12$; $t = 30$; $KMnO_4$)

Fig. 11A–C. Details of cells, showing protrusions of the electron dense membranes into the vacuole (Fig. **A** and **B**) ($D = 0.12$; $t = 30$; $KMnO_4$). Figure **C** represents a detail of a freeze-fractured cell showing a vacuolar vesicle, enfolded by numerous membranous layers ($D = 0.07$; $t = 30$)

ments which thus may be considered as autophagosomes (Holtzman 1976, Fig. 8). Glutaraldehyde/OsO_4 fixation of spheroplasts and isolated autophagosomes indicated that sequestration remained restricted to peroxisomes; other cell components like mitochondria, ER and ribosomes were never observed together with the peroxisome inside an autophagosome (Figs, 7, 8).

The delimiting membranes of the autophagosomes lacked ribosomes (Figs. 7, 8). Autophagosomes were supplied with hydrolytic enzymes by the vacuole. This process, which transformed them into autophagic vacuoles (Holtzman 1976), was initiated approximately at the time of development of the first autophagosome, by fragmentation of the central vacuole into a number of vacuolar vesicles (Fig. 9). This was especially evident in cells grown at low growth rates which contained many large peroxisomes together with a relatively small vacuole (compare Fig. 3) and resulted in a number of small vacuoles scattered throughout the cytoplasm. Some of these smaller vacuoles were localized in close proximity to the autophagosomes (Fig. 10). Subsequently, part of the small vacuoles became incorporated into the autophagosomes via sequestration. This process involved the formation of a protrusion of the electron-dense membranes which first penetrated the vacuolar vesicle (Fig. 11A, B) and subsequently folded (Fig. 11C), thus separating a smaller vesicle from the vacuole (Fig. 13). A schematic drawing of this process is shown in Fig. 12. It should be emphasized that sequestration of peroxisomes and the subsequent incorporation of a vacuolar vesicle within this compartment is a rapid and well-controlled process. In cells that were growing at high rates this process was completed for individual peroxisomes within 20 min after the transfer of cells. Invariably one peroxisome and generally also one vacuolar vesicle were sequestered from the cytosol. Similarly also, as described for the development of autophagosomes, the process of incorporation of vacuolar vesicles did not involve a simultaneous sequestration of other cytoplasmic components.

Following the incorporation of a vacuolar vesicle inside an autophagosome the membrane surrounding the vesicle ruptured, thereby exposing the contents of the sequestered cell-compartment (autophagosome) to attack by vacuolar hydrolases. Probably due to contact with the vacuolar fluid, the characteristic peroxisomal crystalloid (Fig. 14) has now disappeared as shown in both ultrathin cryosections (Fig. 15) and thin sections of glutaraldehyde-fixed cells, and the organelles had assumed a rounded shape. Cytochemically, the activities of alcohol oxidase and catalase were no longer demonstrable after disintegration of the crystalloids. In the case of alcohol oxidase this may be due to the dissociation of the prosthetic group of alcohol oxidase from the protein since increasing amounts of FAD were excreted into the extracellular medium during the first hours after transfer of cells to glucose (P. G. Bruinenberg, pers. comm.). Cytochemical experiments confirmed that at least part of the vacuolar fluid

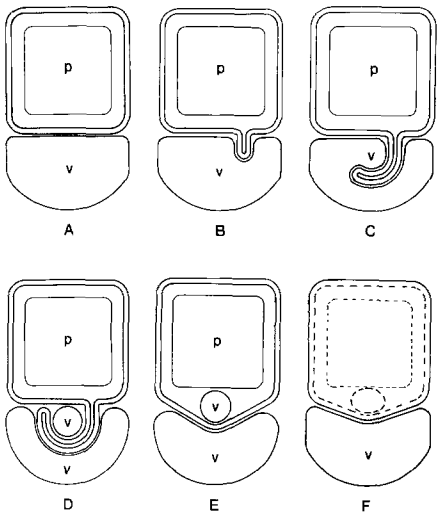


Fig. 12A—F. Schematic drawing of the initial events of peroxisomal breakdown, including its sequestration by the formation of electron-dense membranes (**A**) and the separation and subsequent incorporation of a vacuolar vesicle by means of a protrusion of these membranes (**B—E**). After disruption of the vacuolar vesicle the contents of the sequestered cell compartment are digested (**F**)

was incorporated inside the autophagic vacuoles, since the vacuolar-specific hydrolases, acid phosphatase and glucose-6-phosphatase, which are absent in intact peroxisomes and autophagosomes, were now present in these organelles (Fig. 16). Following the disappearance of the crystalloid, the next stage in the degradation process was characterized by the development of small areas of degradation, as visualized in thin sections of $KMnO_4$-fixed cells, which subsequently increased in volume and number (Veenhuis et al. 1978b, compare also Fig. 20). In addition, the autophagic vacuole was now surrounded by a unit membrane of approximately 90 – 100 Å. The origin of this membrane remained unclear; it probably had derived from the membranes originally surrounding the autophagosome. Attempts to isolate autophagic vacuoles at different stages of development in order to study the transformation of their surrounding membranes have so far failed since we were unable to inhibit the degradation process during the isolation procedure. This procedure generally took 1 – 2 h and in this time interval the degradation of the peroxisomes, which had already initiated at the beginning of the digestion of the cell wall, was completed. This was indicated by electron microscopy which revealed that the fractionation procedures only yielded intact peroxisomes, intact autophagosomes and vacuoles (compare Fig. 8). The vacuolar nature of the latter organelles was established cytochemically; they contained activities of the vacuole-specific enzymes acid phosphatase and glucose-6-phosphatase (Fig. 17) while the presumption that the organelles had originated from autophagic vacuoles was established by the presence of residues of the degradation process in their matrix (Fig. 17). However, in freeze-etch preparations, the fracture-faces of the membranes of such organelles were identical to those of vacuoles of untreated cells (Fig. 18).

In addition to the degradation of peroxisomes by the mechanism described above, a second mode of peroxisomal breakdown was encountered. This process was essentially similar to that described above, but differed in that the peroxisome to be degraded migrated inside a central autophagic vacuole. This latter process was especially apparent in methanol-limited cells which originally contained large numbers of peroxisomes. In such cells the degradation of one or more peroxisomes is initiated by the process described above, thus resulting in the presence of one or more autophagic vacuoles together with a number of peroxisomes still unaffected. Before peroxisomal degradation in the individual autophagic vacuoles is completed, the organelles generally fused (Fig. 19) and thus a large central compartment of autophagic activity was formed in the cells. Uptake of intact peroxisomes by such organelles was again initiated by the formation of a number of electron-dense membranes around these peroxisomes (Fig. 20). Subsequently these membranes fused with the membrane surrounding the central autophagic vacuole resulting in the uptake of the peroxisome into this vacuole (Figs. 21 – 23). A schematic drawing of this process is shown in Fig. 24.

The results obtained after transfer of cells grown in a methanol-limited chemostat at different dilution rates to glucose-containing media revealed that the degradation of peroxisomes did not start at the same time in all the individual cells contained in the culture. Also, not all of the peroxisomes present in one cell were subjected to degradation at the time. In cells from batch cultures taken during the exponential growth phase on methanol, which contain only few peroxisomes, generally the largest organelles were degraded first (Veenhuis et al. 1978b). In chemostat-grown cells on the other hand the largest, mature peroxisomes are localized in the central part of the cells and in such cells the degradation process is generally initiated by the breakdown of one or more of the smaller organelles lying in close proximity to the cell wall (compare Figs. 3 and 4A). Biochemical experiments showed that the rate and extent of inactivation of alcohol oxidase and catalase, which was paralleled by the disappearance of alcohol oxidase and catalase protein, increased with increasing growth rates. Electron microscopical observations revealed that this could be explained by the fact that at low growth rates, due to the increased residence time of cells in the culture, increasing numbers or large 'old' cells were present, recognized for instance by their number of bud scars (Veenhuis et al. 1978a). These contained a considerable number of peroxisomes, which together accounted for up to 80% of the cytoplasmic volume (Veenhuis et al. 1978a). After a shift to glucose media, in such cells degradation of peroxisomes occurred only to a minor extent. This most probably accounted for the relatively high remaining alcohol oxidase and catalase activities observed in cell-free extracts of such cultures 4 h after the shift.

Degradation of peroxisomes was also observed after transfer of cells to media containing glycerol or dihydroxyacetone. Peroxisomal degradation under these conditions was identical to the processes described above for cells transferred from methanol to glucose. However, as was expected from the kinetics of enzyme inactivation, degradation of peroxisimes occurred to a much lesser extent. When deoxyglucose was present in the media instead of glucose, sequestration of individual peroxisomes also occurred (Fig. 25). However, the autophagosomes thus formed remained intact during the following hours of incubation and degradation of their contents was not observed. All autophagic processes, including the formation of autophagosomes, were completely prevented during incubations of methanol-limited cells in glucose media which were kept anaerobic. The latter was also observed in cells transferred to

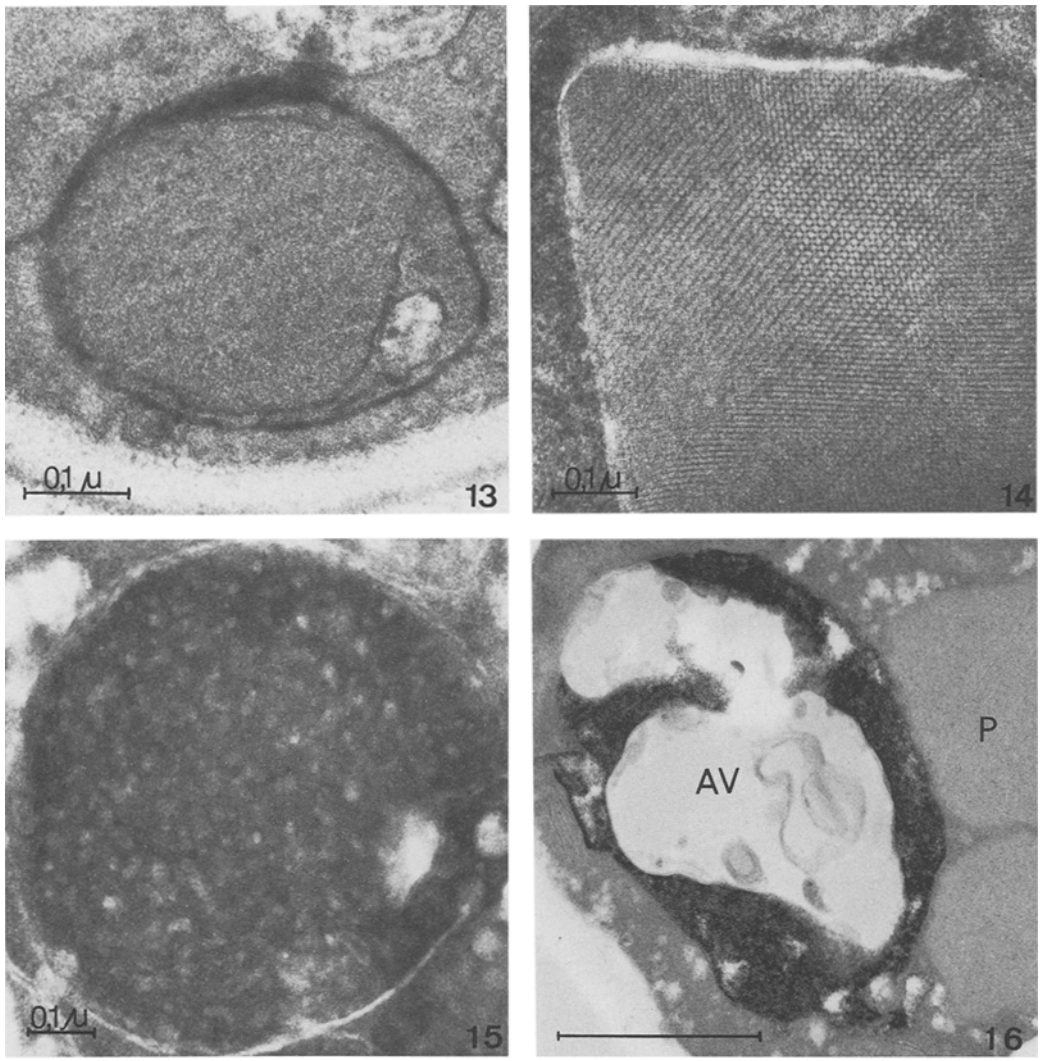
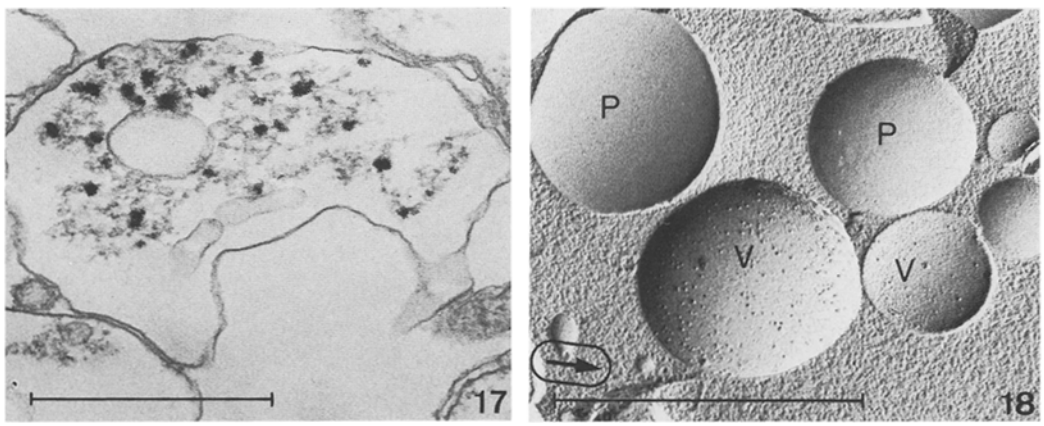


Fig. 13. Section of a cell demonstrating the presence of a vacuolar vesicle within the sequestered cell compartment ($D = 0.07$; $t = 30$; $KMnO_4$)

Figs. 14 and 15. Show details of cryosections of cells, before (Fig. 14) and 45 min after transfer of cells to glucose (Fig. 15). In Fig. 14 the octameric alcohol oxidase molecules are arranged in a crystalloid. This crystalloid is absent in the degrading organelle (Fig. 15); also the characteristic oxidase molecules are no longer recognized ($D = 0.02$; sections stained with uranyl acetate)

Fig. 16. Detail of a spheroplast, incubated with glucose-6-phosphate and $CeCl_3$. The reaction products are present in the autophagic vacuole but not in an intact peroxisome ($D = 0.07$; $t = 60$; glutaraldehyde/OsO_4)



Figs. 17 and 18. Electron micrographs of a subcellular fraction containing residual bodies of isolated autophagic vacuoles and intact peroxisomes. In the residual bodies acid phosphatase activity was demonstrated with $CeCl_3$ and β-glycerophosphate (Fig. 17). After freeze-etching the fracture faces of the membranes surrounding these organelles show the characteristic substructure of normal tonoplasts in contrast to the typical smooth fracture faces of intact peroxisomes ($D = 0.07$; $t =$ glutaraldehyde/OsO_4)

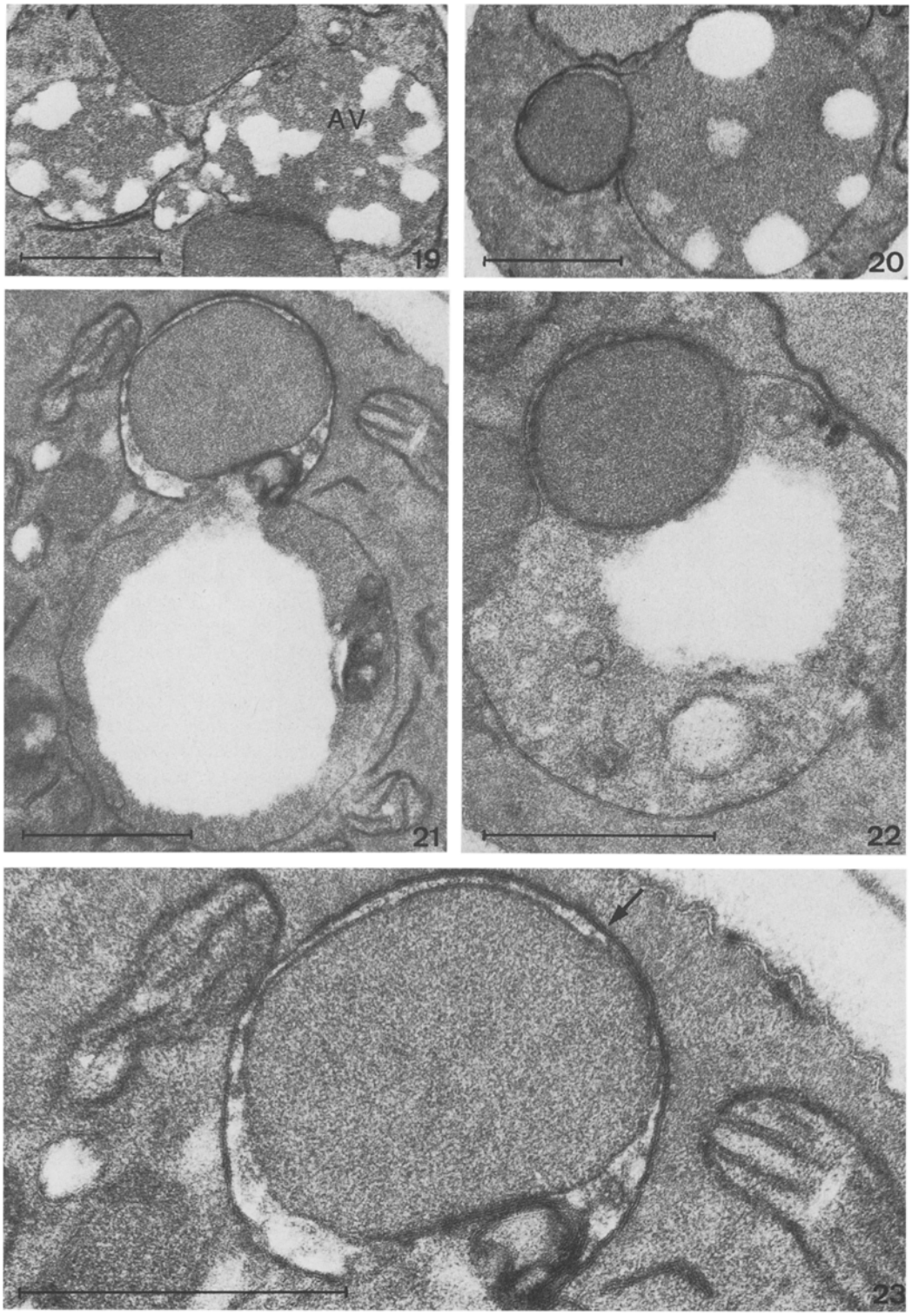


Fig. 19. Detail of a cell showing fusion of two autophagic vacuoles ($D = 0.02$; $t = 60$ $KMnO_4$)

Figs. 20—23. Details of cells, showing different stages of uptake of a peroxisome in an autophagic vacuole. Figure 20 shows the close association of the electron-dense membranes, surrounding an intact peroxisome with membranes of the autophagic vacuole. Figures 21 and 22 show the fusion of these membranes and the migration of the peroxisome inside the autophagic vacuole. Figure 23 is a high magnification, illustrating remnants of electron-dense membranes at the place of fusion. Note that at least two electron-dense membranous layers are separating the organelle from the cytoplasm (arrow). Compare with Fig. 21, showing only one membrane ($D = 0.07$; $t = 90$; $KMnO_4$)

cyanide-containing media. Also under these conditions the sequestration and subsequent degradation of peroxisomes was prevented; in addition the substructure of the peroxisomes, including their crystalline matrix (compare Fig. 14), remained unaffected. These observations confirm the results obtained by HPLC chromatography (see Table 1). Therefore, the observed inactivation of alcohol oxidase in these experiments is most probably caused by cyanide ions which effected the release of its prosthetic group FAD from the holo enzyme.

Discussion

Inactivation of certain enzymes, which occurs when an organism adapts to a major metabolic transition has been termed selective inactivation and may include a change in the physical state of the protein (modification inactivation) or its turnover by proteolysis (degradative inactivation) (Switzer 1977). The experiments described above demonstrated that dependent on environmental conditions both processes may occur in methanol-grown *Hansenula polymorpha* (see also Veenhuis et al. 1979, 1980b, 1983).

A special case of selective inactivation of enzymes involved in carbon metabolism has been oberved in the yeast *Saccharomyces cerevisiae*. After addition of excess glucose to acetate- or ethanol-grown cells, a rapid inactivation of the cytoplasmic enzymes fructose-1,6-bisphosphatase (FBP-ase) and malate dehydrogenase was observed, a phenomenon for which Holzer (1976) proposed the name "catabolite inactivation". Reappearance of enzyme activity in the cells was dependent on *de novo* protein synthesis (Gancedo 1971; Hemmings 1978) and subsequent immunochemical experiments showed that the inactivation of malate dehydrogenase was probably due to selective degradation of enzyme protein (Neeff et al. 1978). Selective inactivation in yeasts which probably also involved proteolysis has also been observed during spore formation in vegetative cells (Betz and Weiser 1976a, b). However, in the above cases the precise in vivo mechanisms of enzyme inactivation remained unknown. Therefore, carbon-catabolite inactivation of alcohol oxidase by glucose as described in the present work, is the first detailed analysis of selective degradative inactivation of enzymes involving extensive hydrolysis of enzyme protein.

In *Hansenula polymorpha* peroxisomes are degraded by means of an autophagic process. Autophagy – as a mode of degradation and turnover of intracellular compounds – is a general phenomenon in a wide variety of eukaryotic cells under normal physiological conditions, but is especially apparent in cells under stress conditions (Glaumann et al. 1980; Holtzman 1976; Matile 1975). In contrast, during exponential growth of *H. polymorpha* autophagy is uncommon. However, the glucose-induced degradation of peroxisomes in methanol-grown cells of this yeast was invariably preceeded by their sequestration from the cytosol; other possible modes, as for instance migration into the central

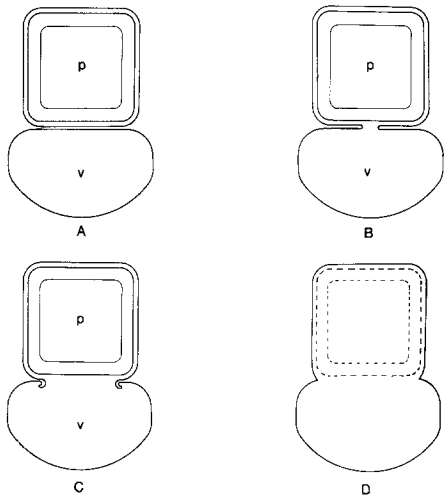


Fig. 24A—D. Schematic drawing of the migration of an intact peroxisome into a central vacuole (or autophagic vacuole) as a result of fusion of the electron-dense membranes with the vacuolar membrane

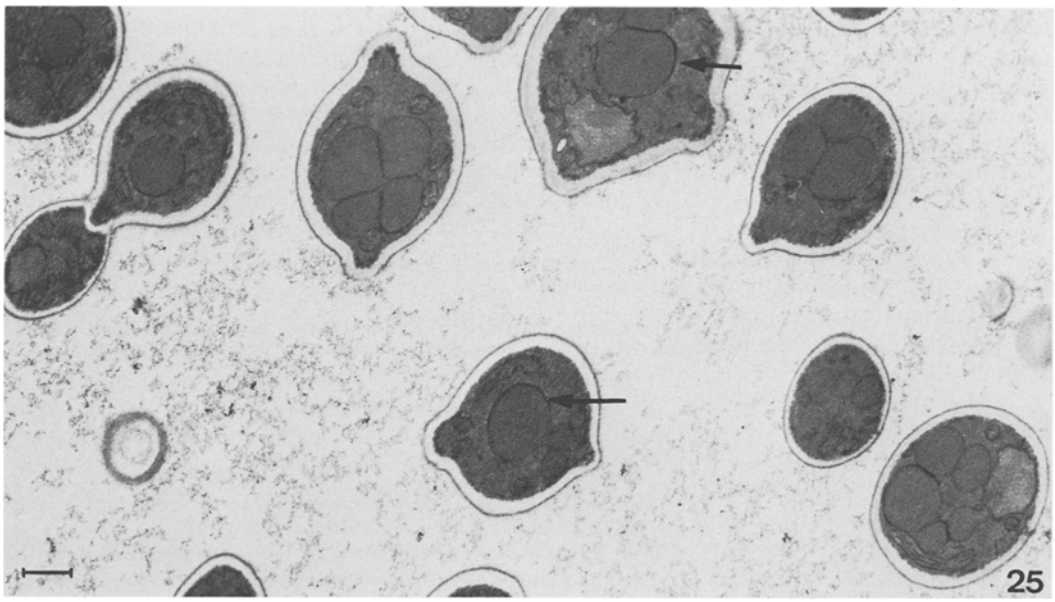


Fig. 25. Survey of cells incubated in deoxyglucose-containing media, showing the sequestration of individual peroxisomes present in such cells (arrows) ($D = 0.12$; $t = 45$; $KMnO_4$)

vacuole by pinocytosis or atrophy, as reported for the degradation of peroxisomes in the fat body of insects during the transition from larva to pupa (Locke and McMahon 1971) were not detected.

Autophagy generally involves a rather random turnover of cell components due to the mode of formation of autophagic vacuoles. This involves the bulk envelopment of cytoplasm by pre-existing cytomembranes such as ER, Golgi or cell membrane (Glaumann et al. 1980; Holtzmann 1976; Matile 1975). However, in *H. polymorpha* membranes surrounding peroxisomes were newly formed; enwrapping of peroxisomes by existing membranes was not observed and is also difficult to envisage in the case of sequestration of one single organelle from a cluster of peroxisomes (see Fig. 3). An additional difference with common autophagy (Glaumann et al. 1980; Holzer 1976) is the high degree of selectivity of this process in *H. polymorpha*. The electron microscopical finding that sequestration – and subsequent degradation – was confined to peroxisomes without the additional turnover of random amounts of cytoplasm is in agreement with biochemical observations. Inactivation was restricted to the – peroxisomal – enzymes alcohol oxidase and catalase; the other enzymes involved in methanol dissimilation, namely formaldehyde and formate dehydrogenases which are of cytosolic origin (Van Dijken 1976), were not inactivated upon transfer of cells to glucose. The observed loss in activity of these enzymes could be accounted for by dilution of enzyme protein over newly formed cells (Egli et al. 1980).

Apart from enzymes involved in carbon metabolism, also enzymes involved in nitrogen metabolism of yeasts may undergo selective inactivation. In *Candida utilis* and *S. cerevisiae*, for instance, NADP-dependent glutamate dehydrogenase, present in high levels during exponential growth on glucose and ammoniumsulphate, was rapidly inactivated after exhaustion of the carbon source; this was paralleled by an irreversible loss of enzyme protein (Hemmings 1978; Mazón 1978). However, in *H. polymorpha* repression of the synthesis of amine oxidase, a key enzyme in nitrogen metabolism during growth on methylamine as the sole nitrogen source and localized in peroxisomes, did not lead to inactivation of this enzyme or to degradation of peroxisomes (Veenhuis et al. 1981a). Similar results have been obtained after repression – by ammonium ions – of other peroxisomal enzymes involved in oxidative nitrogen metabolism, namely urate oxidase and D-amino oxidase. Also in these experiments the decrease in enzyme activity was accounted for by dilution of enzyme protein over newly formed cells (Zwart 1983).

The electron microscopical observations showed that degradation of peroxisomes was dependent on an initial fusion of – at least part of – the vacuole with the sequestered, enzyme-containing, cell-compartment. In yeasts, the vacuole is known as the principal cellular compartment of proteolytic activity (Wiemken et al. 1979; Wolf and Holzer 1978) and there is evidence that peroxisomal enzymes can indeed be hydrolysed by vacuolar proteases (Bormann 1980). In the cytoplasm of yeast cells specific inhibitors of the different vacuolar proteinases are present (Wolf and Holzer 1978). Judged from the high rate of proteolysis, such inhibitors are probably not included in the developing autophagic vacuoles during the process of peroxisomal sequestration and the subsequent incorporation of the vacuolar vesicles. In fact, the observed very tight sequestration of individual peroxisomes may be a mechanism whose function is to avoid the incorporation of cytosolic inhibitors of proteolytic activity. In addition it would prevent unnecessary proteolysis of other cell components since it is difficult to envisage how, for instance in the case of sequestration of several peroxisomes in one compartment, this process could be strictly confined to peroxisomes only.

An early consequence of the release of vacuolar fluid inside the developing autophagic vacuoles included the disruption of the peroxisomal crystalloids. This process was associated by the inactivation of alcohol oxidase and catalase in such organelles which probably involved dissociation of the prosthetic group FAD (Bruinenberg et al. 1982) or the heme moiety. Thus, catabolite inactivation of alcohol oxidase and catalase by glucose may include two processes, namely an initial modification inactivation followed by proteolytic degradation of these peroxisomal proteins. Recently, Müller and Holzer (1981) showed that glucose-induced catabolite inactivation of cytosolic FBP-ase in *S. cerevisiae* also is a two-step process. The first step included a rapid – reversible – inactivation due to phosphorylation of serine residues in the enzyme protein and was followed by a second – irreversible – inactivation due to proteolysis. In contrast, in *H. polymorpha* the first step is irreversible. This was for instance demonstrated in cells of *H. polymorpha* placed under conditions of an enhanced rate of alcohol oxidase synthesis which occurred when methanol-grown cells of this yeast were transferred back to methanol after having been exposed to glucose for 2 h (Veenhuis et al. 1983). The observed rapid recovery of enzyme activity was associated with the development of a number of small peroxisomes, derived from intact peroxisomes which had escaped degradation. However, degradation of those peroxisomes in which the process had already initiated before transfer of cells back to methanol, continued and was completed during further cultivation of cells. Therefore in such cells both the development and turnover of qualitatively identical organelles proceeded at the same time. Similar observations were made after transfer of *H. polymorpha* cells from methanol/methylamine to glucose/methylamine media (Veenhuis et al. 1981a). Therefore, catabolite inactivation of alcohol oxidase in *H. polymorpha* by glucose is considered an irreversible process. Recovery of alcohol oxidase activity, as well as activities of other peroxisomal enzymes which may be present in the organelles (i.e. amine oxidase), is dependent on *de novo* synthesis of enzyme protein (Bormann 1980; Veenhuis et al. 1981a, 1983).

The molecular mechanisms behing the degradation process remain unknown. However, the experiments including transfer of cells to media containing excess deoxyglucose suggested that this energy-dependent process (Bormann 1980) is not solely triggered by the sequestration of peroxisomes.

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